



## Antimicrobial activity and acetylcholinesterase inhibition of novel synthesized pyrimidine derivatives versus *Candida albicans* trafficking to brain and kidney



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### ABSTRACT

The expedient fungi *Candida albicans* (*C. albicans*) is able to thrive in many host niches including blood stream, skin, mucosal surfaces, and different body organs. Herein, the assessment of novel synthesized pyrimidine derivatives as anti fungal agent was investigated. Female albino mice were injected intraperitoneally by *C. albicans* ( $1.5 \times 10^6$  CFU). Infected mice then subjected to treatment with two different doses which were low (50 mg/kg) and high one (200 mg/kg) of diflucan in addition to the newly synthetic compounds (2-(4-(Pyridine-2-yl)aminosulfonyl phenylamino)-6-(naphthalene-2-yl)-4-(pyridine-2-yl)-n-3 carbonitril) and (2-(4-(Pyrimidine-2-yl)aminosulfonyl phenylamino)-6-(naphthalene-2-yl)-4-(pyridine-2-yl)pyridine-3-carbonitril) denoted as (C1 & C2, respectively). Three weeks later gene expression of renal alpha smooth muscle actin ( $\alpha$ -SMA) and cyclooxygenase-2 (COX-2) protein expression were assessed as well as serum malondialdehyde (MDA) and total antioxidant capacity in both kidney and brain tissues. Furthermore, acetylcholinesterase activity was assessed. *Candida albicans* significantly elevated serum MDA. On the other hand, *C. albicans* injection revealed a significant reduction in total antioxidant capacity in kidney as well as in brain tissue. Furthermore, acetylcholine assessment declared a significant elevation. All biochemical parameters were modulated upon new synthesized compounds treatment. Molecular analyses declared a significant down-regulation in renal  $\alpha$ -smooth muscle actin gene expression in addition to, a significant down-regulation in COX-2 protein expression. From data recorded, it could be concluded that, C2 in a dose 200 mg/kg noticeably declared a significant effect comparing with the other treated groups revealing its promising effect as anti-fungal agent.

### 1. Introduction

*Candida albicans* is pathogenic fungi that cause infection to human. These infections may be at oral, vaginal, and systemic, often serious infections in immune-compromised patients [1]. The kidneys and brain are the essential target tissues for *C. albicans* [2–4].

Azole antifungal drugs interfere with fungi metabolism. Recently, resistant strains to these drugs appeared [5]. These drugs may cause cellular oxidative stress including an imbalance of different physiological aspects [6,7].

Cytokines affect renal cells that they induce proliferation and modify their phenotypes. These cells over-express of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and increase collagen production in addition to other components of extracellular matrix [8]. Therefore, the increase of  $\alpha$ -SMA expression by renal cells is related to renal disease progression. Previously, it was suggested that expression of renal  $\alpha$ -SMA may be

associated with progression of renal injury [9]. Moreover, it was confirmed where the interstitial over-expression of  $\alpha$ -SMA is associated with renal frailer [10].

Regulated process in kidney diseases needs vascular smooth muscle cells proliferation into osteoblast cells depending on inflammatory cytokines over-expression in addition to reactive oxygen species production [11,12]. The important functions of cytokines during inflammation especially through the induction of chemokines secretion by endothelial cells and the release of neutrophils into the blood [13].

Cytokines affect renal cells by inducing proliferation and modifying their phenotypes. Interconnection alia COX – 2 and *C. albicans* fungi was previously inspected [14].

*C. albicans* may be carried through bloodstream to brain. Although brain infection by candida doesn't be obvious in adult people, brain infection as meningitis by *C. albicans* is considered as a major snag in newborn [15,16].

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Due to high cost and resistance problem associated with candidiasis to the commonly utilized antifungal drugs [17]. The current study investigated the safety margin of the novel synthesized compounds (C1 & C2) that were previously synthesized and characterized [18] for brain and renal tissues to compare their toxicity with diflucan which is commonly used antifungal drug.

## 2. Material and methods

### 2.1. Chemicals

Diflucan tablets were obtained from Pfizer Company, Egypt. Kits and all hired chemicals were of high analytical grade.

### 2.2. Characterization of compounds C1 and C2

C1 and C2 compounds were previously synthesized and characterized. Furthermore, its antifungal effect was observed in previous vitro study [18].

### 2.3. *C. albicans* separation

*C. albicans* of strain [ATCC36082; Egyptian Type Culture Collection, National Research Centre] was used throughout the study and was isolated as mentioned in [19].

### 2.4. Animals

Female albino mice, weighed (18–22 g m) from National Research Center animal house (NRC). Animals were kept at normalized conditions. According to ethics of Animal Care and use Committee of NRC.

### 2.5. Experimental design

Animals were divided into eight groups (10 mice each)

Group 1; Mice injected with [DMSO] and behold to be healthy negative control group. G2 to 8: Injected by *C. albicans*, intraperitoneally (0.5 ml of  $1.5 \times 10^6$  organisms/ml). After 48 h, the following design was subjected for concessive three weeks [19]. Group 2; Animals were left un-treated and behold as + ve control *C. albicans* infected mice. Group 3; the infected mice administrated diflucan orally (50 mg/kg/day) [20]. Group 4; Mice were treated by diflucan orally (200 mg/kg/day). Group 5; Infected animals were subjected to treatment with C1 compound orally (50 mg/kg/day) [18]. Group 6: Animals undergoes for oral treatment with C1 (200 mg/kg/day) [18]. Group 7; Infected mice undergoes for oral treatment with C2 (50 mg/kg/day) [18]. Group 8; Infected animals that subjected to treatment for C2 (200 mg/kg/day) [18].

Note: All previously mentioned treated regimens were dissolved in 5% DMSO of total administrated volume.

### 2.6. Sample preparation

At the end of the experiment, mice were sacrificed and blood samples were taken from each by aperture of the sublingual vein into sterilizing tubes then let standing for 10 min to be clot. Serum was isolated by centrifugation at 3000 rpm for 15 min then preserved at  $-80^\circ\text{C}$  for biochemical analyses. At the same time, Brain and kidney tissues were removed and rinsed with cold saline (0.9% sodium chloride). Tissue was homogenized in 50 mM phosphate buffer, pH 7.4 (1:5 w/v) then centrifuged at 3000 rpm for 10 min at  $4^\circ\text{C}$ . The supernatant was also stored at  $-80^\circ\text{C}$  for subsequent biochemical analysis.

### 2.7. Biochemical analysis

Serum Malondialdehyde (MDA): was estimated using colorimetric

kits supplied from (Randox Company – Germany) [21].

[Total antioxidant capacity (TAC)] level: It was estimated in both kidney and brain tissues according to manufacturer's instructions using Randox kits [22].

[Acetylcholinesterase]: It was estimated in brain tissue. Acetylcholinesterase activity is carried out based on Ellman's method using Biovision kits [USA, CA 95035].

### 2.8. Real Time- PCR (RT-PCR) analysis

Real-time PCR was used to detect gene expression regulation within a specific forward [5'-GCATCCACGAAACCACCTA-3] and reverse [5'-CAGGAGTAACAAATCAAAGC-3'] primers for ( $\alpha$ -SMA) in the presence of  $\beta$ -actin house keeping gene with forward [5'-TGGAATCCTGTGGCATCCATGAAAC-3] and reverse [5'-TAAAACGCAGCTCAGTAACA GTCCG-3'] primer sequences. Total RNA was extracted from kidney tissue using isolation of total RNA kits (Promega, Madison, WI). Then, the extracted RNA transcribed reversely into cDNA and subjected into a real time PCR system (Stratagene, USA) using SYBR green RT-PCR kits (qiagene- Germany).

### 2.9. Western blotting for COX-2

Kidney homogenate was subjected to SDS-PAGE gel. These proteins were separated then relocated into membranes of nitrocellulose. The membranes then blocked with 5% dry milk in phosphate buffered saline with 0.1% Tween20 then incubated overnight at  $4^\circ\text{C}$  with primary COX-2 antibody (Cell Signaling Technology, Beverly, MA, USA) [23].

### 2.10. Statistical analysis

Row data were analyzed and termed as means  $\pm$  S E M. Statistics was completed by GraphPad Istat-3 program (GraphPad Inc., San Diego, CA). One way ANOVA analysis was outright by S P S S 16 program. Significance setting standard was at  $p < 0.05$ .

## 3. Results

### 3.1. *C. albicans* viability in kidney and brain tissues

As demonstrated in Figs. 1 & 2, *C. albicans* count in both kidney and brain tissues was high in a significant manner in the + ve control group as compared to healthy group. After the treatment with diflucan, C1 and C2 in different doses, proliferation of *C. albicans* was limited. Furthermore, kidney tissue demonstrated that C2 group (group 8) the most effective compound in reducing *C. albicans* counting. Moreover, C2 treated group in a dose 200 mg / kg: group 8) almost reached the

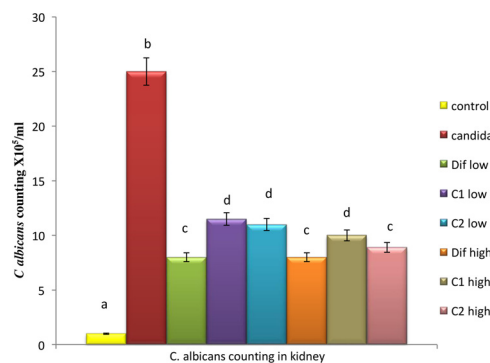
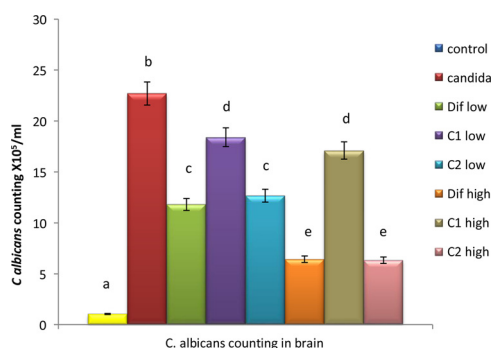


Fig. 1. Effect of different doses of Diflucan, C1 and C2 on *C. albicans* viability in kidney tissue. Data are expressed as means  $\pm$  SEM (n = 10).  $p < 0.05$  is considered significant. Groups having the same letter are not significantly different from each other, while those having different letters are significantly different from each other.



**Fig. 2.** Effect of different doses of Diflucan, C1 and C2 on *C. albicans* viability in brain tissue. Data are expressed as means  $\pm$  SEM (n = 10).  $p < 0.05$  is considered significant. Groups having the same letter are not significantly different from each other, while those having different letters are significantly different from each other.

value of the diflucan treated group. In brain homogenate, both diflucan and C2 in a high dose declared the most pronounced effect in mitigation of *C. albicans* counting.

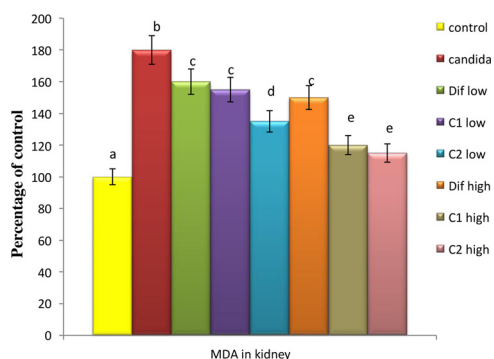
### 3.2. Oxidative stress biomarker modulation in kidney and brain tissues

Infection by *C. albicans* induced an oxidative stress declared by an elevation in serum-MDA level reaching 216.8% comparing to -ve control group (Fig. 3). Diflucan, C1 and C2 administration, declared a significant lowering in MDA value as comparing to + ve control group. Clearly, diflucan and C2 in a dose 200 mg/kg reduced MDA by nearly 2-fold relative to + ve control groups, declaring the most announced action.

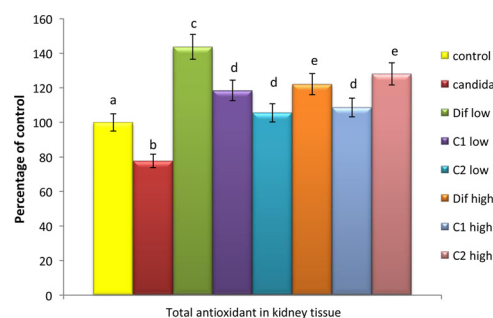
As compared with control group, it was obvious that *Candida* intoxication induced an oxidative stress that elevated total antioxidant capacity significantly in renal homogenate as well as brain tissue (Figs. 4 & 5). This intoxication was receding to be almost near healthy group after treatment. On the other hand, it was obviously in brain tissue that treatment by low dose of diflucan, declared non significant regress of total antioxidant capacity as compared to + ve control group.

### 3.3. Acetylcholinestrase mitigation in brain

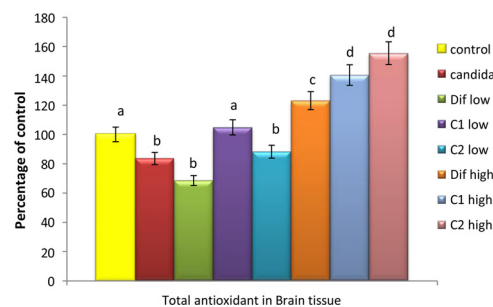
As declared in Fig. 6, acetylcholinestrase value was increased in a significant manner in + ve control group, reaching 300% as comparing to -ve controls. Furthermore, diflucan, C1 and C2 supplementation modulated acetylcholinestrase levels significantly comparing to + ve control group. This modulation was almost near to healthy group values



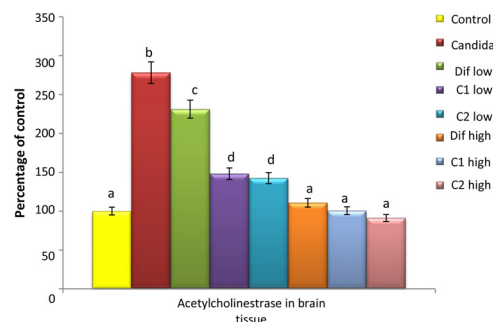
**Fig. 3.** Effect of different doses of Diflucan, C1 and C2 on serum malondialdehyde (MDA) level in *C. albicans* infected groups. Data are expressed as means  $\pm$  SEM (n = 10).  $p < 0.05$  is considered significant. Groups having the same letter are not significantly different from each other, while those having different letters are significantly different from each other.



**Fig. 4.** Effect of different doses of Diflucan, C1 and C2 on Total Antioxidant Capacity in kidney tissue. Data are expressed as means  $\pm$  SEM (n = 10).  $p < 0.05$  is considered significant. Groups having the same letter are not significantly different from each other, while those having different letters are significantly different from each other.



**Fig. 5.** Effect of different doses of Diflucan, C1 and C2 on Total Antioxidant Capacity in Brain tissue. Data are expressed as means  $\pm$  SEM (n = 10).  $p < 0.05$  is considered significant. Groups having the same letter are not significantly different from each other, while those having different letters are significantly different from each other.

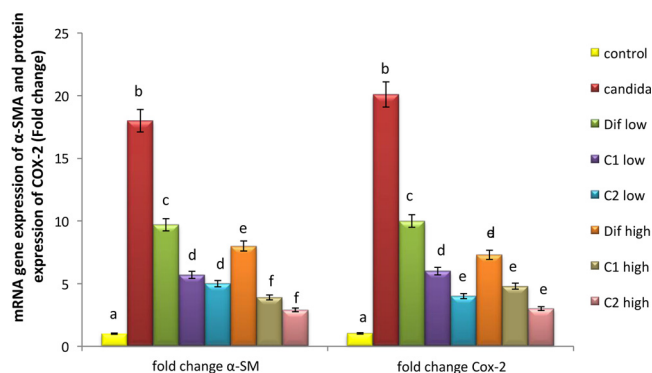


**Fig. 6.** Effect of different doses of Diflucan, C1 and C2 on Acetylcholinestrase in Brain tissue. Data are expressed as means  $\pm$  SEM (n = 10).  $p < 0.05$  is considered significant. Groups having the same letter are not significantly different from each other, while those having different letters are significantly different from each other.

in diflucan, C1 and C2 in a high dose followed by C1 and C2 in a low dose. This mitigation recorded 148% to 100% in all mentioned groups. Moreover, diflucan in a low dose administration was the worst regimen to reduce intoxication induced by *C. albicans* infection in brain by almost 250% as compared to healthy group.

### 3.4. Regulation of renal $\alpha$ -SMA

$\alpha$ -SMA gene expression declared a significant increment in + ve control group, reaching 17.4 fold comparing to the healthy control group. This elevation was significantly down-regulated within C1, C2 and diflucan supplementation. These reductions were amounted to 10, 7 and 5 folds for diflucan, C1 and C2 (50 mg/kg) respectively. Moreover, high doses (200 mg/kg) when administered, the amount of



**Fig. 7.** Effect of different doses of Diflucan, C1 and C2 on  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) gene expression and cyclooxygenase (COX-2) protein expression in *C. albicans* infected groups. Data are expressed as means  $\pm$  SEM (n = 10).  $p < 0.05$  is considered significant. Groups having the same letter are not significantly different from each other, while those having different letters are significantly different from each other.

decrease was amounted to 9, 5 and 3 for diflucan, C1 and C2 respectively (Figs. 7).

### 3.5. Regulation of renal COX-2 protein expression

Injection of *C. albicans* declared a significant increment in the expression of renal Cox-2 protein. Furthermore, administration of diflucan, C1 and C2 down-regulated the expression of COX-2 protein significantly in all treated groups comparing to healthy group (Figs. 7 & 8).

## 4. Discussion

Despite the great evidence that diflucan is the most common antifungal drug, it showed accumulation in the body and resistance problem that induce various inflammations and affect function of different organs. So, study of more effective safe drug is needed. The current study, evaluated novel synthetic anti-fungal compounds in mice model that were previously committed as antifungal agents in vitro in addition, to liver and vagina tissues [18].

Naphthalene nucleus as an active constituent in antifungal drugs inhibits the synthesis of bacterial cell wall by bounding to penicillin bounding proteins (PBPs). It also inhibits certain PBPs that activate bacterial autolytic process. Moreover, pyridines were previously reported as antifungal agents. It inhibits DNA and RNA synthesis, bacterial cell division and dihydropteroate synthase enzyme [24]. In the current study, efficiency of novel synthetic compounds which are conjunction of pyridine and naphthalene (C1 and C2) was investigated to overcome resistance dilemma associated with conventional antifungal medicament. These compounds were previously observed as antifungal agents in vitro [18]. As declared in the current study, *C.*

*albicans* availability was detected and counted in treated groups with different doses of diflucan, C1 and C2 in kidney and brain tissues declaring a significant reduction in *C. albicans* counting as comparing to positive controls. Furthermore, results revealed that new synthetic compounds might be promising in reducing *C. albicans* that trafficking kidney and brain.

The present study declared that *C. albicans* injection increased serum MDA as comparing with the healthy group. However, administration of diflucan, C1 and C2 displayed a significantly decrease in MDA as comparing with *C. albicans* infected group which is inverted their anti-oxidant their antifungal influence as their pyridine nucleus is a concurrent inhibitor of dihydropteroate synthetase enzymes that is essential for folic acid synthesis and required for cells to make nucleic acids, such as DNA or RNA, leading to in cell division inhibition [24].

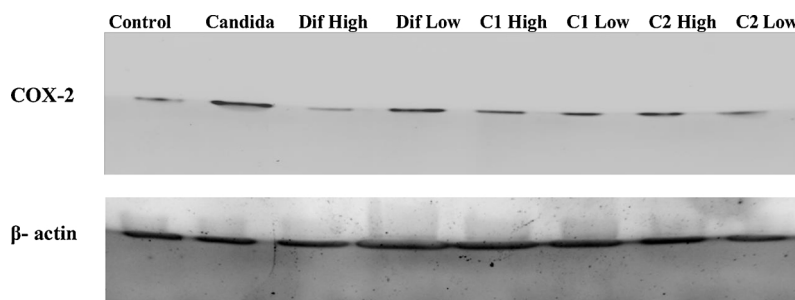
The Present study also declared an obvious reduction in total anti-oxidant capacity in both kidney and brain tissues upon *C. albicans* injection as comparing to healthy group while administration of diflucan, C1 and C2 exhibited a significant elevation in total antioxidant capacity as compared to *C. albicans* injected group. Different studies previously reported that malevolence of *C. albicans* seems to be multifactorial; however, the ability of this fungus to augment the stress resistance is essential to be more survival within host in different organs. Different studies investigated an association of the oxidative effects (resistance degree and oxidative damage induction) and anti-oxidative effects (ability to adapting and induction of anti-oxidative enzymes) [25–27].

During infection, *C. albicans* is transferred by circulatory system to different organs of the body, especially to brain. Here in acetylcholinesterase as an important neurotransmitter was down-regulated upon treatment by diflucan, C1 and C2 with the superiority of C2 as a new promising drug for *C. albicans* treatment that migrate to brain tissue. As previously reported, *C. albicans* may run away to brain causing meningitis [28] affecting specific proteins in brain microvascular endothelial cells [29], Cerebral macroabscess [30]. Moreover, antimicrobial activity in addition to acetylcholinesterase inhibition was previously reported [31].

$\alpha$ -SMA, a marker of kidney myofibroblast, is enhanced by Smads over expression [32].

Herein,  $\alpha$ -SMA mRNA gene expression was significantly up-regulated upon candida intoxication as comparing with healthy group. Appearance of  $\alpha$ -SMA positive cells during renal fibrosis induced by candida injection was previously declared [33]. Furthermore, acute kidney failure was reported to be associated with *C. albicans* infection was also confirmed [34]. Moreover, all treated groups significantly down-regulated with the excellence of C2 especially within 200 mg/kg dose.

Renal COX-2 protein expression was elevated after *Candida* injection was investigated. Previously, it has been demonstrated that COX-2 elevation associated with *candida* infection [35,36]. Furthermore, *Candida* led to over-expression of cytokine genes [37]. These receptors induce inflammatory cytokines and arachidonic acid (AA) from cells of the host that is converted by both cyclooxygenases (COXs) and



**Fig. 8.** Western blot analysis of different doses of Diflucan, C1 and C2 and their effect on renal cyclooxygenase (COX-2) protein expression in *C. albicans* infected groups.



lipoxygenases to eicosanoids [38]. Herein, regulation of expression of COX-2 protein with the superiority of C2 (200 mg/kg) was investigated that indicates the efficiency of C2 as anti-fungal promising drug. Here in, the effectiveness of the current compounds (C1 & C2) may be due to the combination of naphthalene and pyridine nucleus. Furthermore in a previous study, antimicrobial activity was investigated for these compounds [18,39].

## 5. Conclusion

C2 in a dose 200 mg/kg noticeably showed the most significant effect in the down-regulation of the oxidative stress in addition to acetylcholinesterase inhibition,  $\alpha$ -SMA gene expression and COX-2 protein expression induced via *C. albicans* infection and declared a significant effect comparing with the other treated groups revealing its promising effect as antifungal agent.

## Conflict of interest

The authors have no conflicts of interest to declare.

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